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Detection of damage in mammalian sperm cells

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Abstract

Ejaculated semen is washed for in vitro fertilization or diluted and processed to allow optimal and long-term low temperature liquid- and cryo-preservation. However, sperm are vulnerable to the washing, dilution, temperature and osmotic changes involved in sperm storage. In this review, a number of techniques are considered for detecting damaged spermatozoa.

Staining protocols have been developed to detect the membrane and organelle integrity of mammalian sperm cells. Plasma membrane integrity is usually assessed after staining cells with membrane-impermeable dyes or alternatively with acetylated membrane (AM) permeable probes that are selectively de-esterified and become membrane impermeable and thus entrapped into viable cells only (AM ester loading). Organelle-specific dyes are commonly used to detect functionality of mitochondria or the acrosome. A distortion in the lateral and bilayer organization of lipids as well as the peroxidation of fatty acid moieties can be quantified and localized in living sperm. The relation of a disordering in the sperm membrane's lipid architecture and sperm deterioration versus capacitation is discussed. Finally, the integrity of sperm DNA can be measured at three different levels by assessing the degree of DNA-protamine condensation, the incidence of breaks and nicks in the DNA and the frequency of fragmentation of the nuclei into sub-haploid apoptotic bodies. The relevance of detecting DNA aberrations and especially the putative link to the incidence of apoptosis is critically considered.
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1. Introduction

Ejaculated sperm is in the middle of its journey towards the oocyte. Sperm is formed in the testis [1,2] and matured in the epididymis [3,4]. After ejaculation sperm should reach the isthmus part of the oviduct at the ipsilateral side where ovulation takes place [5–7]. At this side the sperm that have been activated (capacitation, for review, see [8,10]) may bind to the extracellular vestment of the oocyte (the zona pellucida [10,11]). This zona binding induces the acrosome reaction and the hyper-active motile sperm can drill through the zona pellucida (see [9]). Finally, sperm cells will bind to and fuse with the oolemma [12]. After fertilization the condensed DNA of the sperm is unfolded and a male pronucleus is formed that fuses with the female pronucleus so that a diploid genome unique to the new individual is formed [13,14]. Thus, for a successful conception the fertilizing sperm should have functional competent membranes, organelles and an intact haploid genome. In assisted reproductive techniques (ART), such as artificial insemination (AI) and in vitro fertilization (IVF), sperm is not directly introduced in the female genital tract at ejaculation but first collected in a bottle or tube either by the use of an artificial vagina, by manual stimulation or by electro-ejaculation (for overviews of sperm collection techniques, see [15,16]). The collected sperm is washed, diluted and, for storage purposes, eventually cooled and stored for extended time prior to (i) its introduction in the female genital tract (AI) [15,16], (ii) addition to a fertilization well with a mature oocyte (conventional IVF) [16] and (iii) selection of one morphologically perfect motile sperm for injection into the oocyte (intracytoplasmic sperm injection ICSI) [17,18]. All sperm processing steps may introduce damage to sperm DNA, membranes and organelles. In order to be able to obtain better fertilization results it is necessary to assess the quality of sperm just before their use in ART. This review provides an update of assays developed to monitor deterioration in sperm membranes, organelles and DNA. We have focused on protocols that make use of fluorescent probes measurable in a flow cytometer for high-throughput, objective and repeatable multi-parametric assessment of sperm deterioration. The working principles of the stains used for assessing certain sperm deterioration are reviewed. Finally, the relevance of the fluorescent staining protocols for predicting the quality of semen is discussed.

2. Membrane and organelle integrity

2.1. Plasma membrane

The plasma membrane surrounds the entire sperm cell holding together its organelles and intracellular components and by its semi-permeable features maintains the chemical gradient of ions and other soluble components. Specific plasma membrane proteins facilitate transport of glucose and fructose from the extracellular environment into the sperm [19–21]. These transporters are indispensable energy source substrates: in the mature sperm approximately 90% of ATP is produced by glycolysis (anaerobic) indicating the importance of monosaccharides as substrates for ATP production [22,23]. If the sperm plasma membrane is not functionally intact the sperm is considered deteriorated (dead) and in vivo is not capable to fertilize. The latter is not valid for ICSI

in oocytes where freeze-dried and rehydrated sperm (thus without a functional plasma membrane) can been used to produce off-spring [24–26]. However, for other ART like conventional IVF and AI it is essential to assess plasma membrane integrity of sperm. Plasma membrane integrity is usually assessed after staining cells with membrane-impermeable dyes. Those cells that are capable to exclude these dyes can be considered to be alive. An array of membrane-impermeable fluorescent probes with affinity for DNA is currently used for this purpose. Different probes that work on this principle have been tested for sperm cells each with distinct excitation (ex) and emission (em) properties: Hoechst 33258 (ex/em of 358/488 nm wavelength [27,28]); YoPro-1 (ex/em of 488/515 nm wavelength [29]); propidium iodide [30,31] or ethidium homodimer-1 [32] (both have an ex/em of 488 and 568/>620 nm wavelength); ToPro-3 and TOTO (ex/em of 647/670 nm wavelength [33]). An alternative (or simultaneous) way to study membrane integrity is the use of acylated membrane dyes (AM loading). By virtue of their acetyl moieties these membrane probes are amphipathic and thus can pass the intact membrane and enter the living sperm. Entered probes are immediately deacylated by intracellular esterases leaving the probe membrane impermeable. Thus, living sperm cells will get loaded with probes that become entrapped in the cell, whereas, the entered probes easily leak out of deteriorated cells with damaged membranes. Fluorescein diacetate and carboxy(methyl) derivatives have been used as AM loading probes to stain viable sperm [31,34]. Recently, a new AM loading dye (SYBR-14) has been introduced into spermatology [35,36]. Once entered the living cell, SYBR-14 deacylation also results in binding of this probe to DNA rendering this probe with fluorescent properties (ex/em 488/515 nm wavelength). For principles of each of the above-mentioned DNA binding sperm viability probes, see Fig. 1. It is possible to color living cells with SYBR-14 (green fluorescent) and deteriorated cells with ethidium homodimer or propidium iodide (red fluorescent) cells simultaneously. In fact, such combination of stains is available as the LIVE/DEAD Sperm Viability Kit. This double staining kit for sperm plasma membrane integrity is handy when working with frozen/thawed sperm. The combination of dyes enables flow cytometric staining immediately after freeze-thawing without any laborious processing step (like diluting and centrifuging) that may affect sperm integrity in between [37]. Another neat positive effect is that non-sperm particles, deliberately added to get better cryo-preservation media (like egg-yolk vesicles or lipid particles from milk), do not have to be separated from sperm before flow cytometric assessment of sperm plasma membrane integrity. Those added particles normally interfere with flow cytometric analysis of sperm cells, but since they do not contain DNA they will remain unlabeled for both dyes. In contrast, both living and deteriorated sperm always will pick up one of the two DNA binding probes and the detected fluorescent emission predicts their viability status (see Nagy et al. [37]). An alternative way to test the functionality of sperm membranes is to subject them to a hypo-osmotic environment. For an overview of this so-called hypo-osmotic swelling (HOS) test, see Refs. [38–42].

2.2. Acrosome

The acrosome is a large Golgi/ER derived acidic secretory organelle. It is filled with hydrolytic enzymes that are organized in a kind of enzyme matrix and most enzymes are heavily glycosylated [43]. Initial sperm-zona binding will trigger the acrosome reaction

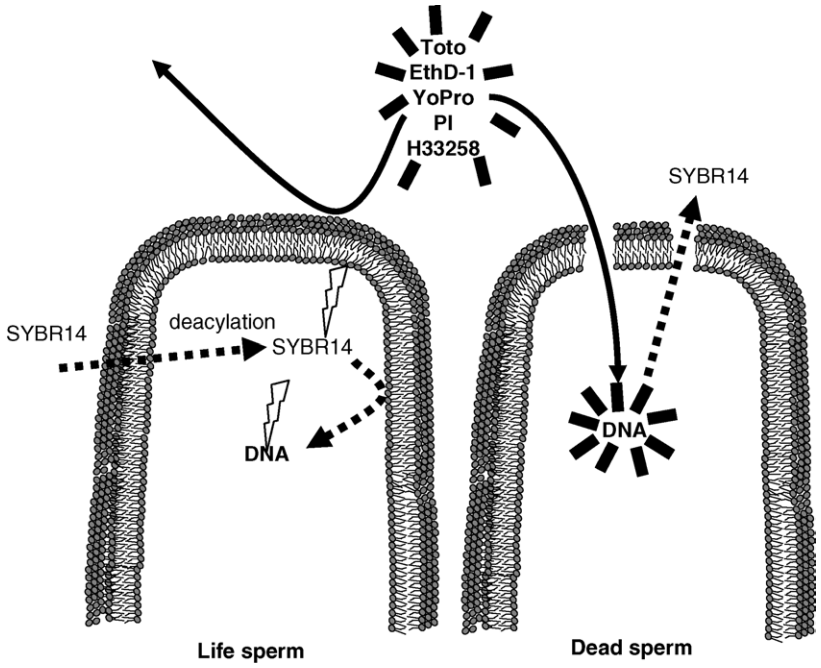

Fig. 1. Discrimination between intact and plasma membrane damaged sperm using DNA affinity probes. DNA of life sperm can be stained with the AM probe SYBR-14. This membrane permeable DNA binding probe is deacylated by intracellular esterases. The deacylated probe cannot diffuse back over the plasma membrane out of sperm. The entrapped fluorescent probe stains the sperm nucleus as it has weak affinity for DNA. In sperm with deteriorated plasma membranes SYBR-14 will leave the cell. DNA of deteriorated sperm can be stained with membrane-impermeable fluorescent DNA staining probes. These probes are not entering life cells but diffuse into the sperm nucleus to bind DNA once the plasma membrane has become deteriorated.

resulting in the release and activation of acrosomal enzymes. This together with the acquired hyper-activated motility will help the sperm to penetrate the zona pellucida [44]. The acrosome must remain intact before and during the transit of the sperm to the isthmus until zona binding has been accomplished. Early acrosome reactions render sperm infertile, and therefore, it makes sense to assess acrosome integrity before ART. Acrosome integrity is commonly measured with fluorescent conjugated lectins. The lectin conjugates bind to specific carbohydrate moieties of glycoproteins that are exclusively localized in the acrosome. Depending on the mammalian species the most commonly used lectin conjugates used are *Pisum sativum* (green pea; PSA), or of *Arachis hypogaea* (peanut; PNA) although for human sperm concanavalin A lectins (conA) can be used as well [45]. The ultra-structural localization of lectin conjugates can be followed after immuno-gold labeling of ultra-thin cryo-sections of sperm. For porcine, equine and canine sperm PNA labeling was specific for the outer acrosomal membrane [46–48], whereas, PSA was labeling acrosomal matrix glycoproteins [48]. Both conA and PSA also bound to some extent to the sperm plasma membrane [48,49]. Acrosome-specific lectins can be conjugated with an array of fluorescent groups. For sperm the use of PNA-FITC (ex/em 488/515 nm wavelength [46]), PNA-TRITC

(ex/em 568/590 nm wavelength [50]) and PNA-RPE (ex/em 488/620 wavelength [51,52]) are described in literature. Detection can be done on living sperm: the absence of fluorescence is indicative for an intact acrosome, and fluorescence is indicative for acrosome disruption or acrosome reaction. This technique is commonly used in flow cytometry [53] or in life cell imaging microscopy [52]. When acrosome staining is carried out on fixed and permeabilized sperm, full fluorescent acrosomes are considered to be intact [32], whereas, acrosomes with lower, patchy or equatorial band fluorescent staining show signs of acrosome disruption or reaction. With the latter technique it is possible to show the degree of acrosome reactions of sperm that have bound to the zona pellucida at a given time [54]. Similar to the lysosome, the acrosome is an acidified organelle with an internal pH of 5. Therefore, specific probes normally used to stain lysosomes, such as Lysotracker Green TM (ex/em 488/515 nm wavelength), can be used to specifically stain the sperm acrosome [55,56]. A variety of other Lysotracker dyes (ex/em Orange TM 488/550, Red TM 568/590, Deep Red TM 628/650 nm wavelengths) are available with different absorption and emission spectra enabling combinations with other dyes for multi-parametric fluorescent assays (see Section 2.4). Antibodies raised against specific intra-acrosomal proteins can also be used for acrosome integrity assessments. The immunolabeling of the inner acrosomal membrane protein CD46 has been described for this purpose for a number of different mammals (for references, see Fig. 2). The principles of acrosome staining methods are summarized in Fig. 2.

2.3. Mitochondria

Sperm mitochondria are localized in the mid-piece area enrolled over the principal part of the flagellum. Mitochondria produce ATP by oxidative phosphorylation. However, the importance of mitochondria for sperm motility (an ATP consuming process) is recently reconsidered. (i) Freshly ejaculated sperm largely produce ATP by glycolysis (>90%) even in buffers that are in contact with air (16% oxygen) [23]. (ii) The ATP-dependent tubulin sliding, responsible for sperm motility, takes place at the distal part of the flagellum in the tail. Thus, ATP consumption is rather far away from the site of aerobic ATP production. (iii) The fibrous sheath of the tail contains enzymes involved in glycolysis (anaerobic ATP production) and knock out mice that fail to express one of these enzymes are immotile [57]. (iv) Sperm subjected to chemical drugs that uncouple oxidative phosphorylation from the electron carrier chain in the inner mitochondrial membrane (IMM) remain motile and alive [22]. (v) Paternal mitochondria are not involved in any event after fertilization and are degraded by the fertilized oocyte [58,59]. Nevertheless, mitochondria are thought to provide the mid-piece and the sperm head with ATP required for housekeeping processes of membranes. Among them one of the most important and ATP consuming housekeeping process is to maintain the Na^+/K^+ gradient over the plasma membrane. The Na^+/K^+-ATPase involved also indirectly drives other transporters and thus regulates the chemical and electric gradient over the plasma membrane. The functional integrity of mitochondria may thus be important for sperm survival in the female genital tract or during ART. Mitochondria can be stained with specific dyes for these organelles (for an overview, see Ref. [60]). Originally, Rhodamine 123 was used to selectively stain functional mitochondria. The principle of this probe is that it only fluoresces red when the proton gradient over the IMM is built up (Rhodamine 123 is a potentiometric membrane dye).

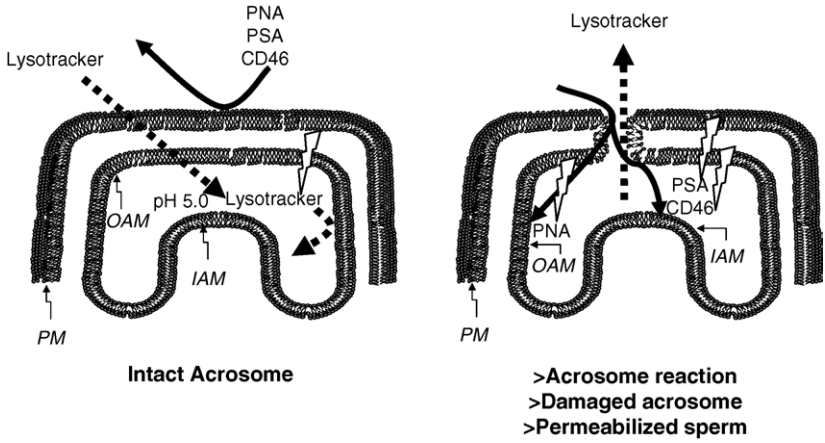

Fig. 2. Discrimination between intact and reacted (or deteriorated) acrosomes. Intact acrosomes can be stained with Lysotracker™ dyes. These dyes fluoresce at pH 5.0 and accumulate in acidic organelles, such as the acrosome. Once the acrosome in not anymore intact the pH becomes neutral and the Lysotracker dye can diffuse out of the acrosome. Lectins (PNA, PSA and others) and antibodies (antiCD46 indicated as CD46) raised against intra-acrosomal (glyco)proteins can be used to discriminate intact and deteriorated/reacted acrosomes either using unfixed sperm or in permeabilized sperm (see Section 2.2). Intact are impermeable for lectins and antibodies, whereas, acrsome reaction or disruption allows entry of those macromolecules. PNA binds to a membrane glycoprotein of the outer acrosomal membrane (OAM), PSA binds to a component of the enzyme matrix in the lumen of the acrosome while antiCD46 binds to a membrane protein of the inner mitochondrial membrane [134,135]. In unfixed cells lectin and antibody staining is indicative for acrosome disruption/reaction. In permeabilized sperm only full fluorescence of the acrosome reflects an intact acrosome and diminished signal indicates the loss of acrosomal content.

This proton gradient is a result of an active electron carrier chain and is coupled to oxidative phosphorylation: the protons flowing back through the ATP synthase to the matrix of the mitochondrium are driving the aerobic ATP production. In unstained mitochondria the proton gradient has collapsed and consequently aerobic ATP production has ceased. Thus, unstained sperm do not contain functional mitochondria, whereas, positive stained cells are aerobically functional [61]. Remarkably, an individual sperm either has a fully fluorescent mid-piece or is not fluorescent indicating that depolarization of the IMM is an orchestrated event occurring at once over the entire mid-piece [22]. The newly developed Mitotracker Deep Red TM (628/650), Mitotracker Red TM (568/595), Mitotracker Orange TM (488/550) and Mitotracker Green TM (488/515) (ex/em in nm wavelengths, respectively) also selectively label the respirating mitochondria and can be used for multi-parametric sperm assessments (see Section 2.4). Mitotracker Orange CM-H_2TMROS (551/576) and Mitotracker X-Rosamine CM-H_2XROS (578/599) (ex/em in nm wavelengths, respectively) become fluorescent after oxidation, a process that only takes place under oxidative respiration. Since this process is only relevant in functional mitochondria these probes are suitable to discriminate sperm with deteriorated mitochondria from aerobically capable sperm [52,62]. For mitochondrial staining principles, see Fig. 3. Recent developments have resulted in new applications for the so-

called Mitotracker dyes. (i) After staining sperm, the suspensions can be fixed and remain stained specifically on mitochondria [63]. (ii) Some of the Mitotracker dyes like JC-1 change their fluorescent properties due to changes in the potential of the IMM (JC-1 switches from orange fluorescence in the aerobic functional mid-piece towards green fluorescent after IMM depolarization) [64,65]. JC-1 can be used to report depolarization of the IMM and thus to report on mitochondrial functionality. The wide spectral range of available fluorescent Mitotracker dyes make these probes applicable for multi-parametric fluorescent assays (see Section 2.4). An alternative way to detect proper ATP production is to observe sperm motility characteristics by computer assisted sperm motility analysis (CASA) (for a recent review, see Ref. [66]).

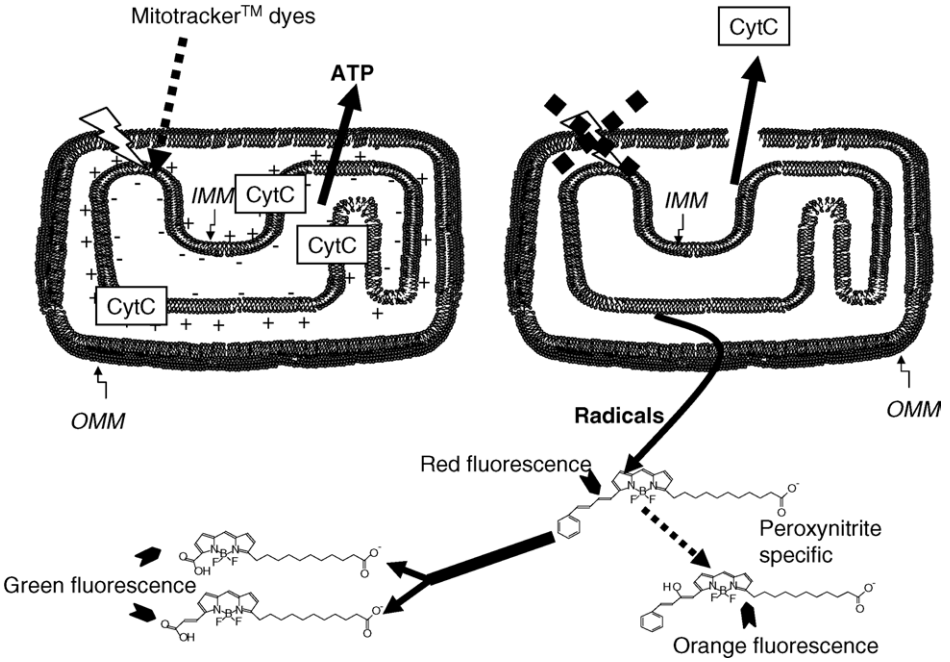

Fig. 3. Detection of respiratory status of sperm mitochondria. As detailed in Section 2.3, aerobe metabolism uses the build up of a proton gradient to couple the electron transport to oxygen (to form water) with the production of ATP by the ATP synthase. The potential of the inner mitochondrial membrane (IMM) can be sensed by potentiometric dyes (Mitotracker™ dyes) that diffuse over the plasma membrane and the outer mitochondrial membrane (OMM) and accumulate in the IMM. Some of the Mitotracker dyes only fluoresce when intercalated in the IMM's potential gradient. Others only fluoresce after interacting with radicals that convert them to fluorescence. For principle of JC-1 staining, see Section 2.3. Once the mitochondria loose their integrity the membrane proton gradient is lost and thus ATP production. Furthermore, cytochrome *C* (CytC; one of the electron transport chain components) is lost and diffuses out of the mitochondrion. As a consequence respiration is blocked and Mitotracker dyes do not sense the IMM potential. Radicals are no longer kept within the matrix of the mitochondria but can oxidize environmental molecules. C_{11}BODIPY$^{581/591}$ (see Section 2.6) can be used to detect lipid peroxidation in the sperm plasma membrane. The red fluorescent probe can peroxidize into the three structures indicated resulting in a green shift in the probe's emission properties.

2.4. Multiple sperm parameter staining

Obviously, the color palette available for preparation of fluorescent conjugates of proteins (for instance, lectins and antibodies) and for each of the mentioned types of fluorescent sperm integrity probes allows multi-parametric sperm staining [37]. For example, it is possible to stain sperm cells simultaneously with four dyes to discriminate: (i) viable sperm from plasma membrane deteriorated sperm (using PI and SYBR-14), (ii) acrosome integrity using PNA-PE, (iii) functional status of mitochondria using Mitotracker Deep Red TM and (iv) non-sperm events [37]. The wide range of spectral variations of each class of membrane and organelle probes makes it possible to design assays to detect multi-parametric features of sperm deterioration. The sperm properties can be measured after staining frozen/thawed sperm immediately after thawing. No washing and fixation steps are required and very accurate data can be read within 10–20 s when using a flow cytometer equipped with a blue (argon 488 nm wavelength) and a red (helium/neon 638 nm wavelength) laser at a speed of approximately 10,000 sperm/s.

2.5. Lipid organization

The capacitation status of sperm usually is assessed with the fluorescent antibiotic chlortetracycline (CTC). This membrane probe binds to the sperm plasma membrane in a Ca^{2+}/Mg^{2+} dependent manner thus forming highly fluorescent complexes of CTC with Ca^{2+} and Mg^{2+} ions bound to membranes [67]. In somatic cells the probe can be intracellularly transferred by endocytosis and consequent vesicle mediated membrane transport towards membranes of organelles. Therefore, CTC is used to detect Ca^{2+} ordering to certain organelle membranes [67,68]. Viable sperm cells become labeled with CTC at those parts of the surface membranes where Ca^{2+} is present above a certain threshold concentration to allow CTC immobilization. In intact non-capacitated sperm this results in a plasma membrane confined F-pattern, an overall staining of the sperm head. This pattern changes into a B-pattern for capacitated sperm, a more prominent staining of the apical area of the sperm head and decreased staining at the posterior area of the sperm head [69]. Finally, the AR pattern emerges indicating that sperm has induced the acrosome reaction [70]. Those cells have a characteristic loss of CTC staining at the apical head area probably by the removal of the mixed acrosome plasma membrane vesicles generated during the acrosome reaction. Nowadays, CTC is routinely used to simultaneously assess the incidence of sperm capacitation and acrosome reactions [71] and the staining protocol has been recently adapted for flow cytometric analysis. The flow cytometer can only discriminate different amounts of fluorescence but to a lower degree can monitor the distribution of that fluorescence per sperm particle. It is not clear whether the amount of CTC fluorescence of pattern B differs from pattern F or that only AR patterns show a decrease in CTC staining [72]. We should note that the Ca^{2+} dependency of CTC staining makes the probe useless for discriminating between the calcium dependent and independent pathways leading to capacitation [8,52]. Another interesting phenomenon with respect to the topic of this review is that the capacitation-specific B staining pattern for CTC has also been observed in frozen-thawed sperm [73–77]. Consequently, some authors proclaimed from this finding that freeze-thawing induces a capacitation-like response to

sperm. The phenomenon speculatively was termed cryo-capacitation [78]. Recent published data place this hypothesis under debate [79,80]. Finally, it is noteworthy to mention that sperm can only very briefly be stained with CTC and need to be fixed immediately after this procedure. It remains unclear how the CTC-sensing of Ca^{2+} attachment to sperm membranes relate to the increase in intracellular free calcium supposed to take place during sperm capacitation enabling the acrosome reaction. An alternative method for assessment of capacitation is to stain sperm with the hydrophobic dye Merocyanine 540 (M540). M540 monitors a decreased packing order of phospholipids in the outer leaflet of the plasma membrane lipid bilayer [81,82], which allows its intercalation into the hydrophobic core of the membrane, this believed to occur in capacitated spermatozoa [8,83]. In the pig, capacitation brings about modifications in the surface of the spermatozoa [84], a collapse in the plasma membrane phospholipid asymmetry [29,51,52] and an increase in the ability of live acrosome-intact cells to bind zona pellucida components [85]. Under these conditions, M540 staining also increased [51] and the membrane fluidity changes detected by M540 preceded the changes picked up by CTC. This together with the fact that M540 stains sperm Ca^{2+} independently makes M540 a more valuable stain for evaluating the early and Ca^{2+} dependent events of capacitation [83]. Incorporation techniques that make use of labeled phospholipids analogs or labeled aminophospholipid-binding proteins can also be used to investigate the phospholipid changes occurring in the plasma membrane of spermatozoa. For example, 6-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino-caproyl (C6NBD)-phospholipids can be used to assess the degree of phospholipid asymmetry in the plasma membrane of the spermatozoon. C6NBD-phospholipids are used to show that boar spermatozoa maintain lipid asymmetry in their plasma membranes after ejaculation. The reported asymmetry is similar to that reported for other species of mammalian spermatozoa, as well as for other cell types [86]. Annexin V is a calcium-dependent phosphatidylserine (PS)-binding protein. When conjugated to a fluorochrome, such as FITC, annexin V permits the recognition of cells with exposed PS. In ejaculated sperm, or sperm that does not respond to in vitro capacitation treatments, PS is confined to the cytoplasmic side of the sperm plasma membrane [86]. Similarly, labeled Ro-09-0198 can be used to detect surface exposure of phosphatidylethanolamine (PE). Gadella and co-workers [52,62] used FITC-annexin V and labeled Ro-09-0198 to demonstrate that bicarbonate induces the exposure of PE and PS on the surface of boar stallion and human spermatozoa. The findings were in agreement to those with the C6NBD-phospholipid analogs [51]. Thus, an array of probes can be used to monitor more disordered phospholipid arrangements in the plasma membrane bilayer during sperm capacitation. The principle signaling pathway behind the membrane alterations detected by the above-mentioned membrane probes is summarized in Fig. 4. As mentioned above these changes might equally be linked to initial membrane damage during cryo-preservation [87–89] or to early capacitation-specific membrane alterations [8,51,52]. We should note here that the capacitation-related CTC, M540 and annexin V staining patterns predominantly show up on viable sperm cells if physiological conditions were maintained during experimentation. To guarantee this sperm suspension should be at 38.5 °C under humidified atmosphere and constantly in equilibrium with 5% CO_2 in air. Adaptations should be made to allow equilibration and maintenance of these experimental conditions in the tubing of the flow cytometer and for the slide chamber of the inverted confocal microscopy equipment to get appropriate set ups to do life sperm imaging experiments. Under such conditions

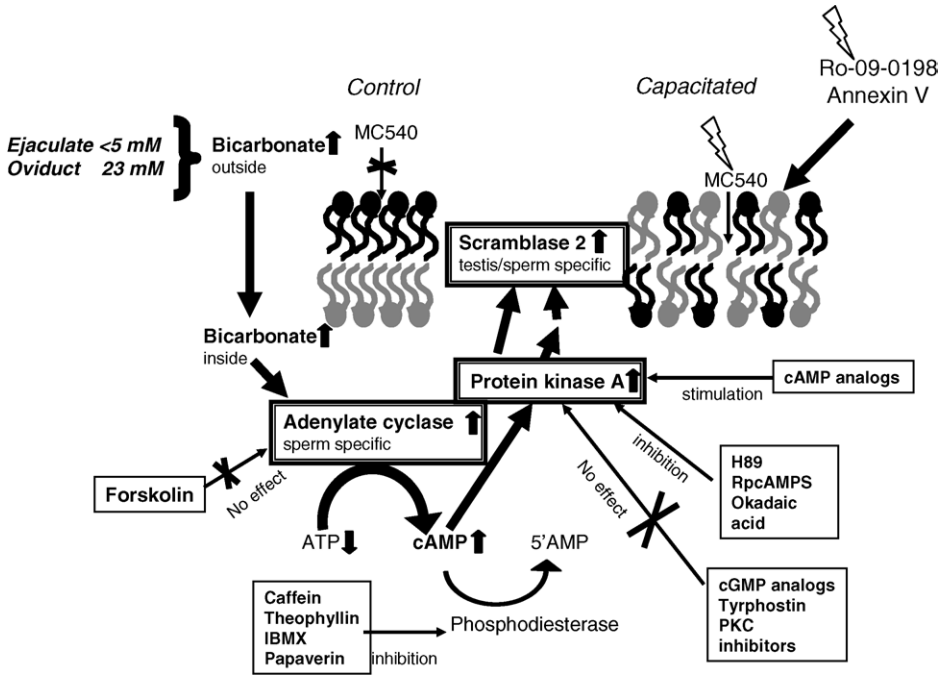

Fig. 4. Sperm signaling resulting in bicarbonate dependent lipid rearrangements in the apical sperm plasma membrane. Elevated bicarbonate levels of the oviduct fluids cause an influx of bicarbonate into the sperm cell. The protein kinase A dependent activation of phospholipids scrambling results in a change in lipid ordering in the sperm plasma membrane. The altered (capacitated) sperm lipid ordering allows MC540 intercalation. Therefore, MC540 can be used to discriminate non-responsive to bicarbonate responsive cells. The exposed aminophospholipids can be stained with Ro-09-0198 (specific for PE) or annexin V (specific for PS). The reordering of the sperm surface can also be assessed with the fluorescent antibiotic chlortetracycline although the principle of this staining is not completely understood. The pathway for lipid scrambling indicated is evidenced by the pharmacological effects of the drugs indicated on this process.

capacitating cells show only marginal deterioration within a period of 4 h incubation in IVF media [52]. In contrast, cooling to even 30 °C will cause membrane disruption in the capacitating sperm leading to cell death [8]. When working with frozen-thawed sperm the ‘‘capacitation-like’’ staining is almost exclusively for deteriorated sperm [88]. These artificial deteriorated cells remain stained and look like capacitated sperm. We have a possible explanation for these phenomena: both incubations cause disordered lipid packing in the sperm membrane. In capacitating cells these changes are required for preparation of the acrosome reaction and the higher fluidity of the sperm membrane at this temperature allows bilayer integrity. Freeze-thawing (and cooling of capacitated sperm) results in a further distortion and collapse of the sperm membranes as the lipid bilayer has become instable by the lowered temperature.

In retrospective we should note here that, as long as, the sperm remains viable every response to an IVF incubation has been considered as a sign of sperm capacitation. However, the relation of these changes to capacitation has not yet been functionally tested.

Do the changes relate: (i) to the ability of sperm to bind to the zona pellucida? (ii) To their priming for the acrosome reaction after this binding? (iii) To acquire hyper-active motility and thus to enable zona penetration? A relation between the detected changes and fertilization characteristics is required. The same is more or less also valid for the tyrosine phosphorylation response occurring in capacitated sperm as well as in frozen-thawed sperm [78,80,90]. As discussed above, membrane changes may alternatively indicate early signs of sperm membrane disruption in capacitating viable cells. Although the same membrane changes and tyrosine phosphorylation patterns have been observed for capacitated and frozen-thawed sperm, the deduced cryo-capacitation concept is under debate and we believe that this theory needs to be experimentally confirmed.

2.6. Lipid peroxidation

In our laboratory, new methods have been developed to assess lipid peroxidation in fresh and frozen stored sperm. Rather than measuring end products of lipid peroxidation (like the thiobarbituric acid reaction, where the formation of malondialdehyde can be detected) our new methods are designed to detect endogenous phospholipid and cholesterol peroxidation in freshly stored and frozen/thawed sperm. Assessments on mass spectrometry allow accurate quantitative and qualitative analysis of the composition of the main phospholipids classes (phosphatidylethanolamine, phosphatidylcholine, sphingomyelin and cholesterol [91]). Furthermore, lipid peroxidation can be followed, quantified and localized after labeling sperm with a lipid peroxidation reporter probe $C_{11}BODIPY^{581/591}$ which is a fluorescent analog for unsaturated fatty acids, the main targets for reactive oxygen species (ROS). Essentially, $C_{11}BODIPY^{581/591}$ changes its fluorescent properties after peroxidation. The intact probe is red fluorescent but turns into green fluorescence when peroxidized by ROS and into orange when peroxidized by peroxynitrite [92]. This green and orange emission shift indicates the presence of reactive oxygen and nitrogen species in the hydrophobic part of lipid bilayers of sperm membranes. The ratio of green + orange fluorescence versus total fluorescence (an indication of the degree of probe peroxidation) correlates well with the degree of endogenous phospholipid peroxidation as measured with mass spectrometry [93]. The degree of probe peroxidation can be followed in particular sperm subpopulations using flow cytometry or localized in individual sperm using life cell imaging microscopy. With the aid of an inverted spectral confocal laser scanning microscope we showed that $C_{11}BODIPY^{581/591}$ is preferentially peroxidized in the mid-piece and (in immature sperm) at cytoplasmic droplets [91,93]. This is probably due to dysfunctions in mitochondrial respiration (see Fig. 3). Furthermore, in our laboratory it is demonstrated that washed frozen-thawed sperm is much less resistant to ROS stress than freshly diluted semen [94]. The relevance of the new lipid peroxidation assays and their implementation in male fertility research remains matter for the future.

3. DNA damage assessments

The integrity of the paternal DNA is of crucial importance for the further development of an embryo. The degree of DNA damage is clearly correlated with the impairment of

embryo development and severe DNA damage cause male infertility [95]. However, IVF experiments with gamma irradiated sperm showed that sperm with severe DNA damage but remained functionally intact at the level of membrane and organelle and motility parameters. In fact, the DNA damaged sperm showed normal ZP binding characteristics and even the fertilization and cleavage rates of the fertilized oocytes remained normal. However, about all four to eight cell embryo stages initiated apoptosis [96–98]. Thus, the reproductive failure, caused by DNA aberrations, appears not at the level of fertilization but at the onset of embryonic DNA expression. DNA damage can be measured at different levels and for sperm DNA mainly three different approaches are currently used.

3.1. DNA condensation

Firstly, the DNA of matured sperm cells (probably the fertilization competent subpopulation) is extremely highly condensed on protamines in a toroid structure. The DNA loops around 500 times around the DNA/protamine toroid structure and extends to approximately 50,000 base pairs per toroid [99]. The sperm's head contains about 50,000 of such structures (for arrangement of DNA-protamine toroids in the sperm head, see Refs. [99–103]). Condensation takes place during spermatid development where histones are removed from nucleosomes by transition nuclear proteins [104–106]. The stripped DNA is coated with protamines and repacked in late-step spermatids in two transition phases [100,107–109]. Proper condensation probably stabilizes the DNA and makes it less vulnerable for oxidative damage. However, repair of DNA damage is not possible in the mature sperm [110]. The condensation status of individual sperm cells can be assessed using transmission electron microscopy (TEM). Condensed nuclei appear homogeneously black in contrast to non-condensed nuclei [111,112] and this appearance in TEM is achieved only in late spermatids during the last steps prior to spermiation. Using a single-cell DNA gel electrophoresis assay (COMET) discrimination can be made between fluorescently labeled DNA of normally condensed sperm nuclei (minimal migration) and more loosely packed DNA (tailing of DNA) after allowing DNA migration on an agarose gel under an electric field. This tailing of DNA was noted for mice with low expression of protamine 2 showing the protamine dependency for late spermatid DNA condensation [113]. Protamines are the key proteins involved in final condensation of sperm DNA [101–105,114,115]. Chromomycin A3 (ex/em of 440 and 470 nm wavelength) can be used to follow the last compaction steps of DNA to protamines. This fluorescent dye binds to deprotaminated DNA (at GC specific regions) but fails to do this after protamination of DNA [116]. The probe is used to detect protamination defects in sperm [117]. The incidence of chromomycin A3 staining on human sperm appears to correlate with the incidence of sperm nicks [118] (see Section 3.2).

3.2. DNA breaks and nicks

The second level is to detect whether sperm DNA is double stranded (intact) or whether single stranded DNA (damaged, for example, in nicks) are formed. Acridine orange can be used to stain single stranded DNA (red fluorescent) and double stranded DNA (green fluorescent). The degree of red acridine orange staining of sperm DNA has been showed to

correlate with male infertility in the sperm chromatin structure assay (SCSA, see the contribution in this volume of Dr. Evenson, for further literature, see Refs. [119,120]). Another method to detect DNA damage is to allow enzymatic incorporation of fluorescent nucleotide analogs by a terminal nucleotide transferease into single stranded DNA areas. TUNEL: fluorescent sperm cells contain single stranded DNA that were labeled by dUTP nick-end labeling (TUNEL) at the 3-OH termini [86,95,121,122]. The proportion of TUNEL$^+$ cells appears to correlate well with decreased pregnancy rates using ART [122]. The proportion of TUNEL$^+$ cells may increase after cryo-preservation [88] although this is contradicted by Duru et al. [123]. Interestingly, the mature and motile human sperm fraction resulting from a swim-up procedure only contained <0.5% TUNEL$^+$ cells [124] indicating that DNA damage is limited in functional spermatozoa. Long-term intensive exposure of sperm to UV light does induce DNA strands and breaks (see Fig. 5). Irrespective of DNA damage, sperm will remain capable to fertilize and activate the oocyte followed by the first cleavages. However, embryo development is blocked after reaching the four to eight cell stages [97,98]. In situ translation of fluorescent nucleotide to nicks with a template dependent DNA polymerase (ISNT) has not been used as alternative to TUNEL to detect DNA damage in sperm cells.

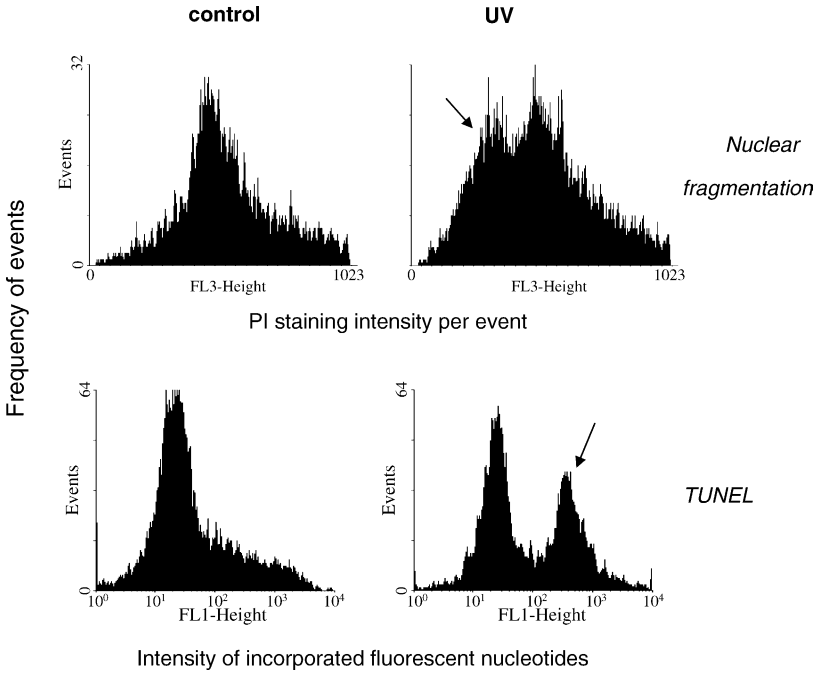

Fig. 5. Detection of DNA damage in human sperm by an hour UV light illumination. Histograms showing the relative distribution of the amount of PI staining (nuclear fragmentation) or amount of fluorescent nucleotide incorporation (TUNEL) per sperm are indicated. Left panels represent control sperm whereas right panels are the sperm stained after UV exposure. The arrow indicates the fragmented sperm nuclei (upper panel) or incorporation of fluorescent nucleotides (lower panel) both resulting from the UV light induced DNA damage.

Another method currently available is estimation of DNA damage, translated by the presence of 8-oxoguanine, as a result of free radical attack [125–127]. The assay is based on addition of a fluorescent labeled 8-oxoguanine binding protein to fixed cells. The yellow fluorescence indicates the presence of 8-oxoguanine. Although this assay is not yet widely used in sperm it may prove a useful tool in ART. COMET (see Section 3.1) and TUNEL assays correlate well with the amount of DNA oxidation and all three responses can be induced by reactive oxygen species [126,128].

3.3. Nuclear fragmentation

The third level to detect DNA damage is to look at nuclear/DNA fragmentation (the latter is defined as a result from double strand breaks in DNA. DNA fragmentation is (unlike the so far mentioned DNA damage) supposed to be specific for apoptosis. DNA fragmentation in apoptotic cells is elicited by effector caspases 3, 6 and 7 that become activated by the caspase cascade during the execution phase of this process (for reviews [129,130]). Briefly, the apoptotic nucleus condenses and falls apart into apoptotic bodies due to caspase 6 mediated breakdown of the nuclear lamina (proteolysis of lamin) and to other effector capsases involved in degrading PARP and in activating domain nucleases and DNases. The resulting nuclear fragments can be imaged by TEM or a fluorescent microscope after staining the sperm with a DNA probe. The double strand breaks generated during cell apoptosis by caspase activated DNases (CAD) are situated between the histones at the so-called interhistone DNA regions. CAD is activated by the effector caspases 3 and 7 that become activated by the apoptotic machinery. DNA from apoptotic cells will after loading and electrophoretic separation appear as a typical DNA ladder on an agarose gel when illuminated with UV light. The discrete DNA bands represent the amount of nucleosomes that were holding the DNA fragment. This DNA ladder is specific for apoptotic somatic cells. We may note that $TUNEL^+$ staining is not specific for cell apoptotis and can also be detected during cell necrosis. In somatic cells the chromatin breakdown is accompanied with the chromatin condensation into apoptotic bodies representing nuclear fragments and this process can be visualized under EM or by fluorescent DNA staining. However, the super-compression of DNA in mature sperm makes further condensation spatially impossible (see Section 3.1) and caspase-mediated breakdown of the sperm DNA has so far not been described. In presence of Triton X 100 (a detergent that disrupts sperm membranes) and 20 mM $MgCl_2$ endogenous sperm DNases actively breakdown the proportion of human sperm DNA that links the DNA-protamine toroids and this process can be stimulated by the addition of DNase I [9]. However, the DNA-protamine toroids remain intact during such experiments. Thus, DNase treated sperm nuclei fall apart into multimeres of 50 kbp DNA-protamine toroids rather than the apoptosis-specific histone-DNA multimeres. It is questionable whether this inter-toroid DNA breakdown takes place during sperm deterioration. When instant (freeze-dried) sperm is rehydrated it will have severe membrane damage but can successfully be used for ICSI [24,25,131–133]. Nevertheless, nuclear fragmentation has been observed in ejaculated human sperm and can be induced by UV light illumination of sperm suspensions (see Fig. 5 or Ref. [62]). We believe that either the non-condensed DNA in aberrant (immature) sperm is selectively destructed by this UV irradiation or that irradiation selectively disrupts DNA segments that are connecting the DNA-protamine toroids.

4. Conclusions

Sperm deterioration can be measured at the membrane and organelle level and an increase of sperm with compromised membranes/organelles will lead to reduced fertility rates. Nevertheless, freeze-dried and dehydrated sperm (i.e. without functional membranes) can still be used for ICSI as DNA remains condensed and intact during such treatment. Sperm deterioration at the DNA level may not affect fertilization rates as long as sperm membranes and organelles remain functionally intact. However, the DNA damage leads to a reduced embryo development after the onset of the embryonic genome. Thus, on one hand, intact functional sperm membranes are essential to achieve fertilization in vivo, but do not essentially contribute to later processes after conception. On the other hand, sperm DNA has no function in achieving fertilization but becomes importantly involved in embryonic development from the onset of embryonic DNA expression (i.e. after the first cleavages). Thus, both deterioration of sperm membranes and DNA are probably important factors involved in male subfertility or infertility. The hypothetical concepts of sperm apoptosis and sperm cryo-capacitation should be reconsidered and experimentally further tested. In this light, special attention should be paid to the unique molecular organization and functioning of sperm membranes and DNA condensation, respectively. Finally, objective high-throughput multi-parameter sperm assessments (preferably with the aid of flow cytometric detection of fluorescent staining) provide statistically stronger data which will be required for future studies to be able to get correlations between sperm quality parameters and fertility results. This may lead to industrial applications, such as selecting top male animals with respect to high quality sperm for breeding programs.

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